11). Accordingly, in normal hematopoietic precursors (Fig. 4E, left), expression of essential (yet to be identified) genes for proper intracellular pathways [such as those involved in important checkpoint controls (*5*, *12*)] may be positively regulated by HEB (or other E proteins) through its promoter interactions with E-box elements (either as homodimers or as heterodimers with cognate partners), through its associations with p300/CBP HATs, and through the resulting cooperative interactions with adjacent promoter-bound activators. In contrast, in t(8;21) cells (Fig. 4E, right), expression of these genes may be silenced because of a dominant interaction of HEB with AML1-ETO that precludes promoter occupancy by p300/CBP but facilitates occupancy by HDAC-containing complexes. Inhibition of these gene expression events may thus predispose cells to further leukemogenic events, possibly as a result of dysregulated checkpoint control.

Beyond defining E proteins as AML1-ETO/ETO targets, our studies also elucidate an E protein silencing mechanism that is fundamentally different from that associated with Id proteins (inhibitors of DNA binding/differentiation) (*13*). Thus, although Id interactions with DNA binding regions of E proteins passively block corresponding promoter interactions, ETO/AML1-ETO interactions with AD1 of promoter-bound E proteins effect a silencing by directing an exchange of cofactors (HATs versus HDACs) that are recruited to target promoters. Like ETO, ETO-related proteins MTGR1 and ETO-2 similarly interact with and inhibit the function of E proteins (*7*). This mechanism may underlie a previously described context-dependent repressive function of the E protein AD1 domain and an enhancer-specific E protein activity (*14*).

E proteins (class A bHLH proteins) are ubiquitously expressed transcription factors that play key roles in the regulation of cell growth and differentiation and programmed cell death (*5*, *6*, *8*, *15*, *16*). E2A is essential for early B cell differentiation events and is a potential tumor suppressor (*6*, *15*). HEB has been implicated in both myogenesis and hematopoiesis (*5*, *17*). Fusions involving E2A (*5*) and HEB (*18*) AD1 domains are associated with leukemogenesis or tumorigenesis. Moreover, inhibition of E protein function by Id proteins negatively regulates cell differentiation and induces proliferation (*13*), an event whose dysregulation is often associated with oncogenesis. Similarly, and consistent with dysregulation of E protein functions by AML1-ETO, it has been shown that AML1-ETO directly induces aberrant hematopoietic cell proliferation (*19*), promotes extensive expansion and self-renewal of human hematopoietic stem cells (*20*–*22*) (the physiological target of many acute myeloid leukemias), and inhibits maturation of multiple lymphohematopoietic lineages (*23*), but is by itself insufficient for leukemogenesis (*24*). These observations further strengthen the idea that E proteins are major physiological targets of AML1-ETO in t(8;21) leukemogenic cells. Our results lead to the hypothesis that there are E protein target genes whose dysregulation by AML1-ETO may be important for t(8;21) leukemogenesis, and they set the stage for identification of these genes and for analyses of the structural basis of the underlying, newly defined regulatory factor interactions.

References and Notes

1. J. D. Licht, *Oncogene* **20**, 5660 (2001).
2. J. M. Scandura, P. Boccuni, J. Cammenga, S. D. Nimer, *Oncogene* **21**, 3422 (2002).
3. B. A. Hug, M. A. Lazar, *Oncogene* **23**, 4270 (2004).
4. I. Kitabayashi *et al.*, *Mol. Cell. Biol.* **18**, 846 (1998).
5. M. W. Quong, W. J. Romanow, C. Murre, *Annu. Rev. Immunol.* **20**, 301 (2002).
6. M. E. Massari, C. Murre, *Mol. Cell. Biol.* **20**, 429 (2000).
7. J. Zhang, R. G. Roeder, unpublished data.
8. A. B. Lassar *et al.*, *Cell* **66**, 305 (1991).
9. D. M. Heery, E. Kalkhoven, S. Hoare, M. G. Parker, *Nature* **387**, 733 (1997).
10. H. Miyoshi *et al.*, *EMBO J.* **12**, 2715 (1993).
11. F. Calabi, R. Pannell, G. Pavloska, *Mol. Cell. Biol.* **21**, 5658 (2001).
12. I. Engel, C. Murre, *EMBO J.* **23**, 202 (2004).
13. M. B. Ruzinova, R. Benezra, *Trends Cell Biol.* **13**, 410 (2003).
14. M. Markus, Z. Du, R. Benezra, *J. Biol. Chem.* **277**, 6469 (2002).
15. I. Engel, C. Murre, *Proc. Natl. Acad. Sci. U.S.A.* **96**, 996 (1999).
16. A. Pagliuca, P. Gallo, P. De Luca, L. Lania, *Cancer Res.* **60**, 1376 (2000).
17. J. S. Hu, E. N. Olson, R. E. Kingston, *Mol. Cell. Biol.* **12**, 1031 (1992).
18. H. Sjogren *et al.*, *Cancer Res.* **60**, 6832 (2000).
19. T. Okuda *et al.*, *Blood* **91**, 3134 (1998).
20. B. A. Hug, S. Y. Lee, E. L. Kinsler, J. Zhang, M. A. Lazar, *Cancer Res.* **62**, 2906 (2002).
21. J. C. Mulloy *et al.*, *Blood* **99**, 15 (2002).
22. C. G. de Guzman *et al.*, *Mol. Cell. Biol.* **22**, 5506 (2002).
23. M. Schwieger *et al.*, *J. Exp. Med.* **196**, 1227 (2002).
24. K. L. Rhoades *et al.*, *Blood* **96**, 2108 (2000).

25. Acknowledgements are presented in the supporting online material. Molecular interaction data have been deposited in the Biomolecular Interaction Network Database with accession codes 150973, 150974, 150975, 150976, 150977, 150978, 150979, 150980, 150981, 150982, 150983, 150984, 150985, 150986, 150987, 150988, 150989, 150990, 150991, 150992, and 150993.

Supporting Online Material

www.sciencemag.org/cgi/content/full/305/5688/1286/DC1
Materials and Methods
SOM Text
Figs. S1 to S7
References and Notes

16 March 2004; accepted 23 July 2004

Small Interfering RNA–Induced Transcriptional Gene Silencing in Human Cells

Kevin V. Morris,[1*†] Simon W.-L. Chan,[2] Steven E. Jacobsen,[2,3] David J. Looney[1,4]

Small interfering RNA (siRNA) and microRNA silence genes at the transcriptional, posttranscriptional, and/or translational level. Using human tissue culture cells, we show that promoter-directed siRNA inhibits transcription of an integrated, proviral, elongation factor 1 alpha (EF1A) promoter–green fluorescent protein reporter gene and of endogenous EF1A. Silencing was associated with DNA methylation of the targeted sequence, and it required either active transport of siRNA into the nucleus or permeabilization of the nuclear envelope by lentiviral transduction. These results demonstrate that siRNA-directed transcriptional silencing is conserved in mammals, providing a means to inhibit mammalian gene function.

Small 21- to 25-nucleotide RNAs have diverse biological roles in eukaryotes, including transposon silencing and antiviral defense by small interfering RNAs (siRNAs) and developmental gene regulation by microRNAs (miRNAs) (*1*–*3*). siRNAs and miRNAs are processed from double-stranded precursors by the ribonuclease (RNase) III–RNA helicase Dicer (*1*). Argonaute proteins can bind small RNAs and are components of effector complexes that downregulate gene expression by several mechanisms (*4*). Small RNAs with perfect homology to their target can cause specific mRNA cleavage (called RNA interference), whereas those with mismatches to their target mediate translational inhibition (*3*). Small RNA–mediated transcriptional gene silencing was first observed in plants through the use of inverted-

[1]Department of Medicine 0678, Stein Clinical Research Building, Room 302, University of California–San Diego, La Jolla, CA 92093–0678, USA. [2]Department of Molecular, Cell, and Developmental Biology, [3]Molecular Biology Institute, University of California–Los Angeles, Los Angeles, CA 90095–1606, USA. [4]VA San Diego Healthcare System, Infectious Diseases, San Diego, CA 92161, USA.

*Present address: Division of Molecular Biology, Beckman Research Institute, 1450 East Duarte Road, Duarte, CA 91010–3011, USA.

†To whom correspondence should be addressed. E-mail: kmorris@coh.org

repeat transgenes or transgenic viruses to generate siRNAs homologous to a target promoter (*5–7*). Promoter-directed siRNAs also silence transcription in the yeast *Schizosaccharomyces pombe*, and transcriptional silencing in *Drosophila* has been linked to an Argonaute protein (*8–10*). Transcriptional silencing by siRNAs probably reflects genome defense mechanisms that target chromatin modifications to endogenous silent loci such as transposons and repeated sequences (*5, 11–14*).

Although siRNA-induced transcriptional gene silencing has not been reported in mammals, transcription of an antisense RNA has been implicated in gene silencing and DNA methylation (*15, 16*), and the structure of mouse pericentromeric heterochromatin may require an RNA component for its maintenance (*17*). Here we investigate whether siRNA-induced transcriptional gene silencing occurs in human cells.

We chose to target an elongation factor 1 alpha (EF1A) promoter–green fluorescent protein (GFP) reporter gene integrated into the genome of human 293FT cells by transduction with a feline immunodeficiency virus (FIV) vector (*18*). siRNA EF52 is homologous to an EF1A promoter sequence essential for transcription (AAG GTG GCG CGG GGT AAA CTG, −106 to −86 base pairs relative to the transcriptional start site) (*19*). A second siRNA homologous to exon 2 of the GFP coding region was designed to target posttranscriptional mRNA destruction. We transduced 293FT cells with the EF1A-GFP vector, allowed 24 hours for integration, then transfected with either EF52, GFP, or a control siRNA matching the human chemokine receptor CCR5. mRNA and DNA were analyzed 48 hours after siRNA transfection. The siRNA targeting the GFP mRNA transcript reduced expression relative to the control as measured by quantitative, real-time reverse transcription polymerase chain reaction (RT-PCR) (Fig. 1A). Potent inhibition of GFP expression was also seen with siRNA EF52 targeting the EF1A promoter (Fig. 1A, open bars).

Several lines of evidence indicate that inhibition of GFP expression by the promoter-directed EF52 siRNA occurs at the transcriptional level. Transcriptional silencing in mammalian cells is associated with chromatin modifications that include histone deacetylation and cytosine DNA methylation (*20*). Silencing by EF52 siRNA was reversed by treating cells with trichostatin (TSA) and 5-azacytidine (5-azaC), inhibitors of histone deacetylases and DNA methyltransferases, respectively (*20*) (Fig. 1A). These agents did not affect RNA interference of the GFP transcript. We confirmed transcriptional silencing using nuclear run-on analysis, which indicated a 93% reduction in transcriptional initiation from the EF1A-GFP reporter gene in EF52-treated cells (Fig. 1B). A glyceraldehyde-phosphate dehydrogenase (GAPDH) control was unaffected by EF52 siRNA in nuclear run-on and RT-PCR experiments. The GAPDH and CCR5 siRNA controls show that promoter-directed transcriptional silencing is specific (Fig. 1B and fig. S1B).

We performed several control experiments to exclude alternative explanations and to confirm that EF52 siRNA down-regulates EF1A-GFP expression by silencing transcription. To ensure that transcription from the integrated transgenic EF1A promoter initiated in a similar position to endogenous EF1A, we performed RT-PCR (fig. S2) (*19, 21*). Specifically, transcripts containing the FIV rev-responsive element (RRE) upstream of the EF1A promoter were not detected, nor were other possible spliced messages initiating from the FIV long-terminal repeat (table S1 and fig. S2). These results show that siRNA EF52 targeted the transgenic EF1A promoter, not a transcribed region. Although the lentiviral vector used integrates into the chromosome and produces a transcriptionally active transgene within 24 hours (*22*), PCR analysis ensured that neither integration frequency nor total lentiviral DNA was affected by siRNA treatment (fig. S3). Collectively, these results demonstrate that EF52 siRNA targets a promoter region rather than transcribed RNA, does not reduce the number of transgenes, and induces transcriptional gene silencing in human cells.

Transcriptional gene silencing in mammalian cells is often accompanied by cytosine DNA methylation, and de novo DNA methylation in plants is guided by small RNAs (*12, 20, 23*). The EF52 siRNA target within the EF1A promoter contains a restriction site for the methylation-sensitive enzyme HinP1I. When methylated, this site is protected from digestion, and a PCR product spanning it can be amplified. The HinP1I site was unmethylated in genomic DNA from untreated cells and from cells treated with control CCR5 or GFP siRNAs. However, DNA methylation was detected in cells treated with EF52 promoter–directed siRNA (Fig. 2A) (the HinP1I

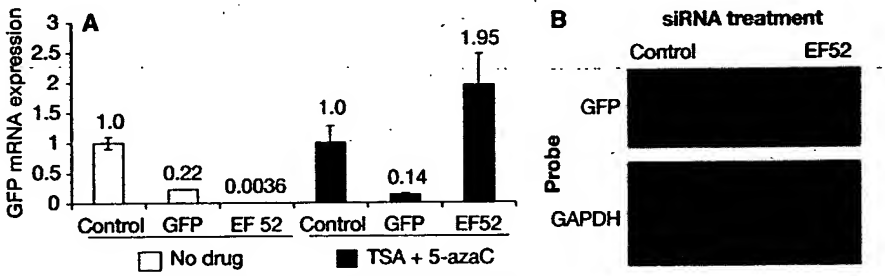

Fig. 1. (A) Promoter-targeted siRNA inhibits gene expression. 293FT cells were transduced in duplicate with lentivirus, then transfected with CCR5 (control), GFP (coding region), or EF52 (promoter) siRNAs. GFP mRNA was quantified by real-time RT-PCR. Gene expression was measured in triplicate; standard deviations are shown. (B) EF52 siRNA silences transcription. Nuclear run-on assays used nuclei from 293FT cells transduced with lentivirus and mock- or EF52 siRNA–transfected.

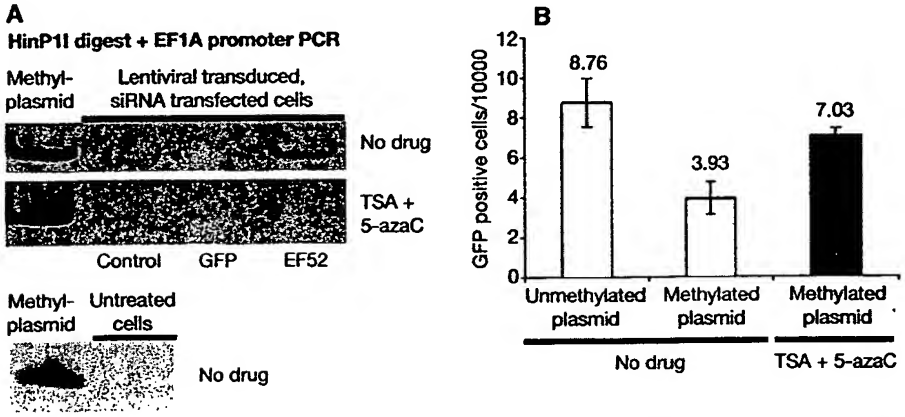

Fig. 2. siRNA-induced transcriptional silencing is associated with DNA methylation. (A) HinP1I-based DNA methylation assay of the EF1A promoter. DNA was prepared either (top) from lentiviral transduced cells transfected with CCR5 control, GFP, or EF52 siRNAs (with or without TSA and 5-azaC treatment) or (bottom) from untreated 293FT cells. HinP1I cut within the EF52 siRNA target site, preventing PCR amplification in unmethylated samples. Sss-I–methylated, EF1A-GFP plasmid DNA was a positive control. (B) Fluorescence-activated cell sorting analysis of GFP expression in cells transfected with Sss-I–methylated, EF1A-GFP plasmid, with or without TSA and 5-azaC treatment. TSA and 5-azaC counteracted transcriptional inhibition caused by DNA methylation. Standard errors of the mean are shown.

assay measured DNA methylation at both the endogenous locus and the EF1A-GFP reporter). Methylation induced by EF52 siRNA was abolished by treatment with TSA and 5-azaC (Fig. 2A). Gene expression from a transfected reporter plasmid was similarly reduced by exogenous methylation (with DNA methyltransferase Sss-I) and restored by TSA and 5-azaC (Fig. 2B). These findings show that siRNA-induced transcriptional silencing in mammalian cells is associated with DNA methylation, a mark of silent chromatin at other loci (*20*).

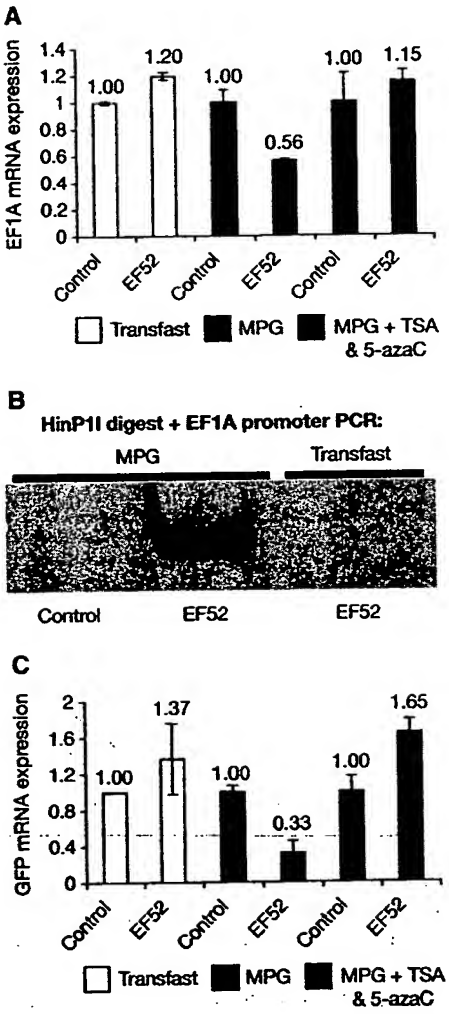

Fig. 3. (A) Promoter-targeted siRNA inhibits endogenous EF1A. EF1A expression was quantified by real-time RT-PCR in cells transfected with control (HIV-1 polymerase) or EF52 siRNAs with either MPG (a nuclear import–mediating peptide) or conventional Transfast reagent. Black columns represent MPG-transfected cells treated with TSA and 5-azaC. Standard errors of the mean were derived from four independent experiments. **(B)** siRNA-induced silencing of the endogenous EF1A promoter is associated with DNA methylation. DNA methylation of the endogenous EF1A promoter was assayed by the HinP1I method in cells that were transfected with control HIV-1 polymerase or EF52 siRNAs with either MPG or Transfast. **(C)** Nuclear-imported siRNAs inhibit transgenic EF1A-GFP long after lentiviral transduction. Lentiviral-transduced cells were sorted for GFP expression, grown for 8 weeks, then transfected as in (A). GFP mRNA expression was measured by real-time RT-PCR. The results represent two experiments with three independent samples per experiment; standard errors of the mean are shown.

It is clearly important whether endogenous EF1A expression is silenced by promoter-directed siRNAs. However, in mammalian cells that have not been transduced with lentivirus, transfected small RNAs lack an efficient nuclear transport mechanism, and mammalian cells have specialized export pathways for hairpin-containing miRNA precursors (*24–26*). This obstacle is circumvented by lentiviral transduction, which permeabilizes the nuclear membrane before siRNA transfection (*22*). In order to assess the effect of siRNA on the endogenous EF1A promoter, we transfected 293FT cells with EF52 and control [human immunodeficiency virus (HIV)–1 polymerase-specific] siRNAs using MPG, a bipartite amphipathic peptide incorporating a fusion peptide from HIV-1 gp41 transmembrane protein and the SV40 virus nuclear localization sequence (*26*). MPG facilitates the nuclear import of nucleic acids, including siRNAs (*26*). Cells transfected with EF52 and MPG showed significantly reduced endogenous EF1A expression by real-time RT-PCR, relative to the control (Fig. 3A). Transfection with EF52 and the conventional Transfast liposome reagent did not cause silencing, despite a transfection efficiency comparable to or greater than that of MPG (Fig. 3A). Silencing in cells transfected with EF52 and MPG was abolished by treatment with TSA and 5-azaC, indicating that it occurs at the transcriptional level (Fig. 3A). Furthermore, HinP1I digestion of the EF1A promoter was blocked in cells treated with EF52 and MPG, but not in cells transfected with EF52 and Transfast (Fig. 3B), indicating that siRNA-induced transcriptional silencing of the endogenous EF1A promoter is associated with DNA methylation.

As the effect of lentiviral transduction on the nuclear membrane is probably transient, we examined the need for nuclear transport of siRNAs in silencing an integrated EF1A-GFP reporter gene well after transduction (Fig. 3C). For this experiment, we transduced 293FT cells, isolated a GFP-positive population after 72 hours, and grew the cells for 8 weeks. As observed for endogenous EF1A, silencing of the integrated EF1A-GFP in this population depended on transfection with MPG and was reversed by TSA and 5-azaC (Fig. 3C). This suggests that siRNA-induced transcriptional silencing of an integrated reporter is not strictly dependent on lentiviral transduction, but rather on the ability of siRNAs to gain access to the nucleus. Silencing of endogenous EF1A and of the integrated reporter gene 8 weeks after transduction was less efficient than silencing of newly integrated EF1A-GFP (compare Fig. 3, A and C, to Fig. 1A). It is possible that MPG is less efficient than lentiviral transduction at transporting siRNAs into the nucleus. Alternatively, newly integrated EF1A-GFP transgenes may be more accessible to siRNAs because of their intrinsic chromatin structure; newly transformed transgenes are more susceptible to de novo DNA methylation and silencing in *Arabidopsis* (*26*).

Transfected siRNAs are generally retained in the cytoplasm of mammalian cells, where they mediate efficient mRNA cleavage but cannot target chromatin (*24*). siRNAs transcribed from hairpin transgenes are exported from the nucleus because they may resemble pre-miRNAs, which are produced by the nuclear RNase III Drosha and cleaved into mature miRNAs by cytoplasmic Dicer (*1*, *3*, *28*). These intrinsic features of mammalian cell biology may indicate why siRNA transport into the nucleus is necessary for transcriptional silencing.

Our findings confirm that siRNA-directed transcriptional gene silencing is conserved in mammalian cells. Small RNAs may guide mammalian transcriptional silencing in many different biological contexts, including the establishment of genomic imprints and targeting of DNA methylation to retroviruses and repeated transgenes (*12*, *29*, *30*).

Note added in proof: After this work was submitted, Fukugawa *et al.* showed that Dicer-defective chicken cells have heterochromatin defects at centromeres, possibly implicating siRNA in centromeric silencing (*31*).

References and Notes

1. M. Tijsterman, R. F. Ketting, R. H. Plasterk, *Annu. Rev. Genet.* 36, 489 (2002).
2. S. I. Grewal, D. Moazed, *Science* 301, 798 (2003).
3. D. P. Bartel, *Cell* 116, 281 (2004).
4. M. A. Carmell, Z. Xuan, M. Q. Zhang, G. J. Hannon, *Genes Dev.* 16, 2733 (2002).
5. M. F. Mette, W. Aufsatz, J. Van der Winden, A. J. M. Matzke, M. A. Matzke, *EMBO J.* 19, 5194 (2000).
6. L. Jones, F. Ratcliff, D. C. Baulcombe, *Curr. Biol.* 11, 747 (2001).
7. T. Sijen *et al.*, *Curr. Biol.* 11, 436 (2001).
8. M. Pal-Bhadra, U. Bhadra, J. A. Birchler, *Mol. Cell* 9, 315 (2002).
9. V. Schramke, R. Allshire, *Science* 301, 1069 (2003).
10. M. Pal-Bhadra *et al.*, *Science* 303, 669 (2004).
11. M. Wassenegger, S. Heimes, L. Reidel, H. L. Sanger, *Cell* 76, 567 (1994).
12. S. W. Chan *et al.*, *Science* 303, 1336 (2004).
13. D. Zilberman, X. Cao, S. E. Jacobsen, *Science* 299, 716 (2003).
14. T. A. Volpe *et al.*, *Science* 297, 1833 (2002).
15. H. Seitz *et al.*, *Nature Genet.* 34, 261 (2003).
16. C. Tufarelli *et al.*, *Nature Genet.* 34, 157 (2003).
17. C. Maison *et al.*, *Nature Genet.* 30, 329 (2002).
18. K. V. Morris, J. Gilbert, F. Wong-Staal, M. Gasmi, D. J. Looney, *Mol. Ther.* 10, 181 (2004).

19. N. Wakabayashi-Ito, S. Nagata, *J. Biol. Chem.* 269, 29831 (1994).
20. A. P. Bird, A. P. Wolffe, *Cell* 99, 451 (1999).
21. T. Uetsuki, A. Naito, S. Nagata, Y. Kaziro, *J. Biol. Chem.* 264, 5791 (1989).
22. M. P. Sherman, W. C. Greene, *Microbes Infect.* 4, 67 (2002).
23. M. Matzke, A. J. Matzke, J. M. Kooter, *Science* 293, 1080 (2001).
24. R. Yi, Y. Qin, I. G. Macara, B. R. Cullen, *Genes Dev.* 17, 3011 (2003).
25. E. Lund, S. Guttinger, A. Calado, J. E. Dahlberg, U. Kutay, *Science* 303, 95 (2004).
26. F. Simeoni, M. C. Morris, F. Heitz, G. Divita, *Nucleic Acids Res.* 31, 2717 (2003).
27. X. Cao, S. E. Jacobsen, *Curr. Biol.* 12, 1138 (2002).
28. Y. Lee *et al.*, *Nature* 425, 415 (2003).
29. X. Cao *et al.*, *Curr. Biol.* 13, 2212 (2003).
30. M. Okano, D. W. Bell, D. A. Haber, E. Li, *Cell* 99, 247 (1999).
31. T. Fukagawa *et al.*, *Nature Cell Biol.* 6, 784 (2004).
32. We thank C. Chung and W. Witke for technical support and K. Suzuki for comments regarding transcriptional gene silencing. S.W.-L.C. is a U.S. Department of Energy (DOE) Energy Biosciences fellow of the Life Sciences Research Foundation. Supported by NIH AIDS training grant no. AI07384, NIH grant no. P01 AI45992 (D.J.L.), the UCSD Center for AIDS Research, NIH grant no. P30 AI36214, and a Beckman Young Investigator Award (S.E.J.).

Supporting Online Material
www.sciencemag.org/cgi/content/full/1101372/DC1
Materials and Methods
Figs. S1 to S3
Table S1
References and Notes

14 June 2004; accepted 26 July 2004
Published online 5 August 2004;
10.1126/science.1101372
Include this information when citing this paper.

Impaired Degradation of Mutant α-Synuclein by Chaperone-Mediated Autophagy

Ana Maria Cuervo,[1]* Leonidas Stefanis,[2] Ross Fredenburg,[3] Peter T. Lansbury,[3] David Sulzer[4]

Aberrant α-synuclein degradation is implicated in Parkinson's disease pathogenesis because the protein accumulates in the Lewy inclusion bodies associated with the disease. Little is known, however, about the pathways by which wild-type α-synuclein is normally degraded. We found that wild-type α-synuclein was selectively translocated into lysosomes for degradation by the chaperone-mediated autophagy pathway. The pathogenic A53T and A30P α-synuclein mutants bound to the receptor for this pathway on the lysosomal membrane, but appeared to act as uptake blockers, inhibiting both their own degradation and that of other substrates. These findings may underlie the toxic gain-of-function by the mutants.

A30P and A53T mutations of α-synuclein, a cytosolic protein that normally exerts a presynaptic function (*1*), cause familial forms of Parkinson's disease (PD) (*2*). Because familial PD mutations in parkin and the ubiquitin carboxy-terminal hydrolase L1 (UCHL1) genes affect the ubiquitin-dependent proteasome proteolytic system, and mutations in the DJ-1 gene (*PARK7* gene on chromosome 1p36) are associated with the closely related sumoylation pathway, proteasomal degradation appears to be involved at least in some PD pathogenic pathways (*3*). Initial reports that α-synuclein is degraded through the proteasome (*4*, *5*) led to the idea that abnormalities in proteasomal degradation of α-synuclein underlie PD (*6*). Some subsequent studies failed to show alteration of α-synuclein levels by proteasomal inhibition (*7*–*9*), suggesting that there are alternate forms of α-synuclein degradation. Whereas proteins with short half-lives are mostly broken down by the proteasome, most cytosolic proteins with long half-lives (>10 hours) are degraded by autophagic pathways within lysosomes (*10*–*12*). Lysosomal inhibitors increase intracellular levels of α-synuclein (*13*–*15*), suggesting that α-synuclein may also be degraded by autophagy. Experimental overexpression of mutant α-synuclein activates macroautophagy, a form of autophagy in which large regions of cytosol are engulfed and trafficked to lysosomes (*12*). Although activation of macroautophagy degrades the mutant proteins (*13*, *16*) and mislocalizes synucleins to autophagic organelles (*17*), inhibition of macroautophagy does not appear to alter the degradation of wild-type α-synuclein (*15*).

In contrast to macroautophagy, a highly specific subset of cytosolic proteins with a motif recognized by the hsc70 chaperone are selectively degraded in lysosomes by a process known as chaperone-mediated autophagy (CMA) (*12*, *18*). Following binding of the chaperone-substrate complex to a lysosomal membrane receptor, lamp2a (*19*), CMA substrate proteins are translocated into the lumen for degradation by hydrolases (*18*, *20*).

We noted that the α-synuclein sequence contains a pentapeptide sequence ($_{95}$VKKDQ$_{99}$) that is consistent with a CMA recognition motif (*21*). In rat ventral midbrain cultures that contain dopaminergic neurons maintained in serum-free medium, we confirmed that the endogenous wild-type α-synuclein exhibited a relatively long half-life (16.8 ± 2 hours; Fig. 1A) (*22*). In contrast to the relatively small effect of epoxomicin, a selective proteasome inhibitor, on the half-life of α-synuclein (a 2.3-hours increase in half-life; Fig. 1A), ammonium chloride, which inhibits lysosomal proteolysis independently of the form of autophagy that delivers substrates to lysosomes, strongly inhibited α-synuclein degradation (9.6-hours increase in half-life; Fig. 1A). As described previously in PC12 cells for human wild-type α-synuclein (*13*), addition of 3-methyladenine, an inhibitor of macroautophagy, did not modify the degradation of rat α-synuclein (16.1 ± 2.4 hours). It thus appears that endogenous rat α-synuclein in ventral midbrain neuronal cultures is degraded in lysosomes but not by macroautophagy. We then examined the degradation of human wild-type α-synuclein expressed in PC12 cells (*16*), in which serum removal activates both macroautophagy and CMA (Fig. 1B). Serum removal markedly enhanced human α-synuclein proteolysis (from a half-life of 33.1 ± 6.3 hours to 19.7 ± 2.1 hours; n = 5), whereas ammonium chloride inhibited its degradation (half-life of 48.9 ± 5.4 hours and 80.3 ± 16.6 hours, in the presence or absence of serum, respectively; n = 5) (Fig. 1B) (supporting online text 1).

The presence of a CMA motif, however, does not guarantee that a protein is degraded by this pathway (*21*). The most direct test of whether a protein is a CMA substrate is to determine its binding, uptake, and degradation in isolated intact lysosomes (*19*, *20*, *23*, *24*). Because synuclein protofibrils have been suggested to destabilize the membranes of synthetic vesicles (*25*), we first confirmed that isolated lysosomes were not disrupted by wild-type or mutant α-synuclein proteins at concentrations as high as 70 μM (fig. S4B) (*22*). Under these conditions, we found that purified α-synuclein added to the incubation medium was translocated into and degraded by intact lysosomes, because lysosomal protease inhibitors increased

[1]Department of Anatomy and Structural Biology, Marion Bessin Liver Research Center, Albert Einstein College of Medicine, Bronx, NY 10461, USA. [2]Neurobiology Laboratory, Foundation of Biomedical Research of the Academy of Athens, Athens 11527, Greece and Department of Neurology and Pathology, Columbia University, New York, NY 10032, USA. [3]Center for Neurologic Diseases, Brigham and Women's Hospital, Department of Neurology, Harvard Medical School, Cambridge, MA 02139, USA. [4]Departments of Psychiatry and Neurology, Columbia University, Department of Neuroscience, New York State Psychiatric Institute, New York, NY 10032, USA.

*To whom correspondence should be addressed. E-mail: amcuervo@aecom.yu.edu